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<b>(21) International Application Number:</b> PCT/US91/01683 <b>(22) International Filing Date:</b> 13 March 1991 (13.03.91) <b>(30) Priority data:</b> 493,511 14 March 1990 (14.03.90) US <b>(71) Applicant:</b> THE IMMUNE RESPONSE CORPORATION [US/US]; 5935 Darwin Court, Carlsbad, CA 92008 (US). <b>(72) Inventors:</b> BOHLEN, Heribert ; Laarbeeklaan 103, B-1090 Brussels (BE). URBAIN, Jacques ; Tienne du Peuthy 1, B-1338 Lasne (BE). VAN CAMP, Benjamin ; THIELEMANS, Kristiaan ; Laarbeeklaan 103, B-1090 Brussels (BE).		<b>(74) Agents:</b> CAMPBELL, Cathryn et al.; Pretty, Schroeder, Brueggemann & Clark, 444 South Flower Street, Suite 2000, Los Angeles, CA 90071 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> IDIOTYPIC VACCINATION AGAINST B CELL LYMPHOMA  <b>(57) Abstract</b>  The invention provides a method of inducing an effective immune response to pathogenic lymphocytes by administering dendritic cells previously pulsed with the idiotype protein of interest. In one embodiment, a method for the active immunization of a mammal against lymphoma is provided. This embodiment comprises exposing dendritic cells to idiotype Ig to make idiotype pulsed dendritic cells and injecting the idiotype pulsed dendritic cells back into the mammal, whereby immunity against lymphoma cells is induced. In another embodiment, the invention relates to the administration of both idiotypic cells and pulsed dendritic cells.		

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## IDIOTYPIC VACCINATION AGAINST B CELL LYMPHOMA

## BACKGROUND OF THE INVENTION

The vertebrate immune system functions to recognize and eliminate materials, such as pathogens, bacteria and viruses, which are recognized as foreign to the host. In addition, the immune system also serves as a surveillance system to eliminate malignant cells, which, because they express altered proteins on the cell surface, are regarded as foreign. Immunological responses to foreign substances, termed antigens, comprise a humoral response and a cellular response. The humoral response involves the production of specific antibodies, or immunoglobulins, which recognize and bind to the antigen. The cellular response involves the proliferation of cells which aid in elimination of the antigens.

An immune response can often be induced or heightened by active immunization with a vaccine (or immunogen) comprising an antigen, or a molecule resembling an antigen. Often it is necessary to provide immune enhancers, termed adjuvants, in addition to the immunogen.

This invention relates generally to the area of active immunization and more specifically to vaccines useful for immunization against lymphomas and to adjuvants useful for changing the magnitude and character of the immune response.

The majority of B lymphoid tumors are characterized by the expression of immunoglobulin (Ig) on the cell membrane. The idiotype (id) of the surface Ig can be regarded as a tumor specific antigen or marker, and has

been used as a target for immunotherapy. Monoclonal anti-id antibodies have been used to study the immunobiology of these tumors and have been used in therapeutic trials as well. The passive administration of anti-id monoclonal antibodies (Mabs) especially of mouse origin has been hampered by a number of problems, however, which have reduced their applicability in clinical usage. Some of these problems are (i) the free antigen in circulation (ii), the emerging immune response against mouse Ig and (iii) the heterogeneity of the tumor cells. Several studies have reported that immunization with idiotypic protein or subfragments can protect the animal against the outgrowth of a plasmacytoma or surface Ig bearing lymphoma. The induced immune response includes anti-idiotypic antibodies as well as T cell dependent immunity. In order to evoke such antibodies and immunity against a challenge with tumor cells, it has been shown repeatedly that it is necessary to couple the idiotypic protein to a strong immunogenic carrier and to present this conjugate in the presence of a strong adjuvant.

Idiotypic heterogeneity has been disclosed during a clinical trial with anti-idiotypic antibodies. After an initial partial response induced by the monoclonal antibody, idiotypic variant tumor cells emerged at the original tumor-site. It is likely that such idiotypic variant tumor cells were already present before the monoclonal antibody treatment, but were allowed to proliferate after the selective removal of the idiotypic positive tumor cells.

Active immunization of animals with syngeneic tumor derived Ig or its subfragments elicits the production of anti-id antibodies and induces protection against a subsequent exposure to tumor cells. In most cases, however, a tumor elicits a response which is too weak or

which appears too late to be of lasting therapeutic value. Therefore, either modified id-Ig or strong non-physiological adjuvants were needed. The use of non-physiological immunogens or non-specific activators is  
5 highly undesirable for use with human patients because of side effects, including the possibility of inducing a polyclonal B cell response which could lead to the development of autoimmune disease.

10 There thus exists a long-felt need for a method of enhancing the immunogenicity of vaccines. Preferably, any adjuvants used in connection with such immunization should provide an immune response of sufficient strength to be therapeutically useful. In particular there exists  
15 a need for a means to effect active immunization of the tumor host with syngeneic Ig so as to elicit an effective polyclonal response. The present invention satisfies this need and provides related advantages as well.

20 SUMMARY OF THE INVENTION

The invention provides a method of inducing an effective immune response to pathogenic lymphocytes by administering dendritic cells previously pulsed with the  
25 idiotype protein of interest. In one embodiment, a method for the active immunization of a mammal against lymphoma is provided. This embodiment comprises exposing dendritic cells to idiotype Ig to make idiotype pulsed dendritic cells and injecting the idiotype pulsed  
30 dendritic cells back into the mammal, whereby immunity against lymphoma cells is induced. In another embodiment, the invention relates to the administration of both idiotypic cells and pulsed dendritic cells.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Tumor immunity induced immunization with  
5 idiotypic-KLH conjugates. C<sub>3</sub>H/He mice were immunized at  
weekly intervals with KLH conjugates of 38C13 IgM (----  
two injections: group II, -0-0- three injections: group  
I, -0-0- 1 injection: group III) or control IgM (-0-0-  
two injections: group IV). One week after the last  
10 immunization, mice were inoculated with 10<sup>2</sup> 38C13 tumor  
cells. The numbers correspond to the experimental groups  
of Table I.

Figure 2. Effect of immunization with idiotype IgM  
15 pulsed dendritic cells (DC) on survival of mice after  
tumor inoculation. C3H/He mice were immunized with  
38IC13 IgM-DC (-0-0- : group V), or control IgM-DC (----  
: group VI) or soluble idiotype protein (-♦-♦-: group  
VII) at day 28 before tumor cell (10<sup>2</sup>) challenge. Mice  
20 received one boost of soluble IgM at day -7. The numbers  
correspond to the experimental groups of Table I.

Figure 3. Comparison of syngeneic anti-idiotypic  
antibodies induced by immunization with 38C13-KLH in  
25 Freund's adjuvant and by 38C13 pulsed DC. Sera from mice  
three (-0-0- : group I) or two (---- : group II) times  
immunized with 38C13 KLH conjugates emulsified in  
Freund's adjuvant or with 38 C13 pulsed DC (-♦-♦-: group  
V) were added to wells pre-coated with 38C13 IgM. The  
30 sera were diluted over 8 wells. Bound antibody was  
detected by addition of enzyme labeled goat anti-mouse  
IgG.

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a method for enhancing the  
5 effect of immunization. Such a method is of particular  
usefulness for immunization in situations where an  
effective immune response is difficult to elicit, as with  
many tumor markers. However, the method is also  
applicable in conjunction with immunization against  
10 various pathogens.

Recently, a dramatic enhancement of an antiviral  
immune response by mouse dendritic cells pulsed in vitro  
with virus or with polyclonal anti-idiotypic antibodies  
15 (Ab<sub>2</sub>) was reported Francotte and Urbain PNAS 82:8149  
(1985). Dendritic cells (DC) have been shown to be  
strong stimulators of immune responses to antigens  
attached to their cell surface. The antigen pulsed DC  
primes the resting T lymphocytes which then deliver the  
20 necessary help to antigen-specific B lymphocytes.

The present invention involves the unexpected  
determination that mouse DC pulsed in vitro with idiotypic  
protein from pathogenic lymphocytes can replace the  
25 immunogenic carrier and the non-physiological adjuvant  
previously thought to be required to elicit an effective  
immune response to pathogenic lymphocytes. As used  
herein, the term "pathogenic lymphocytes" refers either  
to unregulated malignant lymphocytes or to lymphocytes  
30 mounted in an autoimmune response. The term idiotypic  
protein from a pathogenic lymphocyte refers to an  
immunoglobulin or a fragment of an immunoglobulin (FAB)  
bearing an idiotypic epitope.

35 Protection against a subsequent tumor cell dose can  
be obtained by pre-immunization with syngeneic idiotypic  
conjugated to an immunogenic carrier and emulsified in

Freund's adjuvant or with in vitro idiotypic-pulsed DC. The control groups treated with the same number of DC pulsed with an irrelevant IgM or immunized with the same dose of soluble syngeneic 38C13 IgM, did not show a  
5 prolonged survival. These data clearly indicate the enhancing effect of dendritic cells and the idiotypic specific suppression of tumor growth.

C<sub>3</sub>H/He mice were immunized with idiotypic  
10 immunoglobulin M (IgM) from the syngeneic 38C13 lymphoma. Conjugation to an immunogenic carrier protein (keyhole limpet hemocyanin; KLH) and a strong non-physiological immune stimulator (complete Freund's adjuvant; CFA) was required to obtain an idiotypic specific humoral and  
15 cellular immunity and protection against a lethal tumor cell challenge. However, when dendritic cells were used for idiotypic presentation, neither immunogenic carrier nor adjuvant were needed. Dendritic cells, having been pulsed in vitro with unmodified idiotypic protein and re-  
20 injected into the animals, were able to induce significant resistance to subsequent tumor inoculation. Alternatively FAB fragments or synthetic peptides bearing an idiotypic epitope could be used for inoculation. Idiotypic specific T-lymphocytes which proliferated in  
25 response to native 38C13 idiotypic (id) as well as cytotoxic T-lymphocytes were observed in both groups. The cellular immune response was stronger in the dendritic cell-treated animals than in case of 38C13-KLH and complete Freund's adjuvant treatment. Both  
30 immunization methods resulted in long-term survivors without tumor cell escape caused by emergence of idiotypic variants or tumor cell dormancy. Remarkably, after one year, 80% of the mice were still alive.

35 Serum analysis of immunized animals showed that the protective effect was not correlated in a simple way to the serum anti-idiotypic titer. High levels of anti-



idiotype antibodies induced by hyper-immunization with Freund's adjuvant could have modulated the surface Ig of the tumor cells which then could escape destruction by id-specific T cells.

- 5 T cells that proliferated in the presence of idiotype proteins could be demonstrated even more than 3 months after the immunization. These cells, although their phenotype is not known, had a cytotoxic effect on the syngeneic tumor cells.
- 10 The following examples are intended to illustrate but not limit the invention.

#### EXAMPLE I

15 Preparation of Dendritic Cells

- Dendritic cells (DC) were isolated using the method described by Steinman and Cohen, J. Exp. Med. 139:380-397 (1974). Briefly a suspension of spleen cells, free of aggregates or clusters, was suspended in a solution of bovine serum albumin (BSA) ( $\rho = 1,082 \text{ g/cm}^3$ ); Fraction V, (Sigma Chemical Co., St. Louis, MO), at a concentration of  $1 \times 10^8$  cell per ml. A low density BSA solution ( $\rho = 1,060 \text{ g/cm}^3$ ) was layered on top. The tubes were spun to equilibrium at 10,000 g for 30 minutes at 4°C. Floating cells were harvested and washed twice in RPMI 1640 medium. The cells were resuspended in complete medium (RPMI 1640, 5% FCS,  $5 \times 10^{-5} \text{ M}$  2-mercapto-ethanol, penicillin, streptomycin, minimal essential amino acids, and sodium pyruvate) and transferred to plastic petri dishes at a concentration of  $1 \times 10^5$  cell/ml for 3 hours at 37°C - 5% CO<sub>2</sub>. Non-adherent cells were removed by gentle pipetting and the adherent cells were kept for another 16 hours in complete medium. The supernatant containing non-adherent low density cells was used as the

source of DC. The contaminating macrophages were removed during antigen pulsing, since the macrophages tended to re-adhere to the plastic surface while the DC remained non-adherent. The purity of the final cell preparation was examined by scanning EM, transmission EM, immunofluorescence and acridine orange staining. Goat anti-mouse Ig labelled with FITC, and anti-Thy-1.2 labelled to biotin (Becton Dickinson), were used to characterize B and T cells respectively.

Dendritic cells were resuspended in complete medium at a concentration of  $5 \times 10^5$  cells per ml in 24 well flat-bottomed plates (1 ml per well). Fifty microgram of purified 38C13 IgM (kappa) idiotype protein or an irrelevant mouse IgM (kappa) (ABPC - Sigma Chemical Co., St. Louis, MO) was added. The plates were kept at  $37^\circ\text{C}$ , in 5%  $\text{CO}_2$  for 4 to 5 hours. Cells were washed several times in sterile PBS and resuspended at  $2.5 \times 10^5$  cell per ml.

20

## EXAMPLE II

Syngeneic Immunization With Idiotypic Ig

$\text{C}_3\text{H}/\text{He}$  mice were obtained from the Laboratory Animal Center of the Catholic University of Leuven. Balb/c and  $\text{F}_1(\text{C}_3\text{H}/\text{He} \times \text{Balb/c})$  mice were bred at the Laboratory Animal Facility at the Free University of Brussels (VUB). 38C13 is a carcinogen (DMBA) induced B cell tumor of  $\text{C}_3\text{H}$  origin. These tumor cells and the in vitro adapted cell line used in this study express IgM (kappa) on the cell membrane but do not secrete large amounts of Ig. An idiotype IgM (kappa) secreting cell line has been obtained by fusion of the tumor cells with a non-secretory myeloma cell line (P3 X 63 Ag 8.653).

35

Four groups of 10 mice each were immunized with 50  $\mu\text{g}$  idiotypic 38C13 IgM or unrelated ABPC IgM cross-linked

to KLH, emulsified in Freund's complete adjuvant (CFA), incomplete adjuvant (ICFA) or PBS, following a schedule shown in Table 1. One week after the last injection 38C13 tumor cells ( $10^2$  cells/mouse) were injected intraperitoneally. This dose is lethal to 100% of non-treated animals by day 30. The presence of tumor and the day of death were recorded.

Figure 1 shows the protective effect of immunization on survival in a typical experiment. The most effective immunization schedule consisted of 2 administrations of idiotype IgM, once in CFA and once in ICFA (group II). These findings were reproducible. There were no long-term survivors when either an irrelevant IgM-KLH conjugate (group IV) or unconjugated idiotype IgM (group VII) were used.

TABLE I

IMMUNIZATION SCHEDULE				
*Time	I*	II	III	IV
Day 28	-	-	-	-
Day 21	38C-KLH/ CFA i.p.	-	-	-
Day 14	38C-KLH/ ICFA i.p.	38C-KLH/ CFA i.p.	-	ABPC-KLH/ CFA i.p.
Day 7	38C-KLH/ PBS i.v.	38C-KLH/ ICFA i.p.	38C-KLH/ CFA i.p.	ABPC-KLH/ ICFA i.p.

\*Experimental group consisting of 10 mice.

\*Day before the i.p. injection of tumor cells ( $10^2$ ) at day 0.

## EXAMPLE II

Immunization with Idiotypic IgM  
Pulsed Dendritic Cells

5        These groups of 10 each of C<sub>3</sub>H/He mice were injected intraperitoneally with  $5 \times 10^4$  IgM pulsed dendritic cells prepared as in Example I, according to the immunization schedule of Table II.

10

TABLE II

*Time	V	VI	VII
15	Day 28	DC-38C13 i.v.	DC-ABPC i.v.
	Day 21	-	-
	Day 14	-	-
20	Day 7	38C13 i.v.	ABPC i.v.
			38C13 i.v.

Experimental group consisting of 10 mice.

\*Day before the i.p. injection of tumor cells ( $10^2$ ) at day 0.

25        One hundred 38C13 cells were injected intraperitoneally one week after the last injection of antigen. The presence of tumor cells and the day of death were recorded.

30        The protective effect of immunization with idiotypic IgM pulsed dendritic cells is shown in Figure 2. A single injection of idio type pulsed DC's followed by a boost of soluble idio type protein resulted in the same survival after tumor passage as in the experiments using  
35 38C13-KLH conjugates and CFA. Control immunized animals (DC pulsed with irrelevant IgM or idio type protein alone) did not show any protection.

## EXAMPLE IV

Assessment of Humoral and Cellular Immunity

5        To assess the role of humoral immunity, the levels of syngeneic anti-idiotypic antibodies were measured by an IgG specific ELISA prepared as follows.

10        Balb/c mice were immunized with purified idiotypic protein cross-linked with keyhole limpet hemocyanin (KLH) (Calbiochem-Behring, Hoechst) with glutaraldehyde according to the method of Maloney, et al., Hybridoma 4:191-209 (1985), which is incorporated herein by reference. The spleen cells were hybridized to the P3 x 15 63 Ag 8.653 myeloma cell line. The monoclonal antibodies E4 and 8E3 were strongly reactive with 38C13 IgM(kappa), were not inhibitable by normal C<sub>3</sub>H serum and did neither bind to normal spleen cells nor to purified IgM myeloma proteins. A monoclonal anti-idiotypic antibody S5A8, of 20 C<sub>3</sub>H origin and a rat monoclonal antibody R7D7 were purified from ascites fluid by double precipitation with ammonium sulfate (40%) were utilized. Biotin labelling of the antibodies was performed according to methods well known in the art.

25        Before the tumor cell injection, the mice were bled by puncture of the retro-orbital plexus. Sera from individual mice of the same experimental group were pooled. Syngeneic anti-idiotypic antibodies were detected by an ELISA assay as described.

30        The results in Figure 3 indicate high levels of anti-id antibodies in mice immunized with 38C13-KLH and CFA. Much lower levels were detected in sera from animals immunized with DC's, indicating that there was no clear correlation between antibody levels and survival. 35 Three injections with IgM-KLH in CFA resulted in a higher

serum level of anti-id antibodies but a lower survival rate (Figure 1).

Cellular immunity was determined by detecting  
5 idiotypic specific T lymphocytes in the spleen of long-term survivors of both immunization approaches. T cell-enriched splenic cells were cultured in the presence of soluble idio-  
type protein or idiotypic IgM coupled to  
10 sepharose beads for 3 days. Control wells contained splenocytes in IL2 containing medium 10% (v/v) of supernatant of rat spleen cell culture containing 4  $\mu$ g/ml concanavalin A for 24 hours, or an irrelevant IgM  
protein. A suspension of splenic cells from surviving  
15 animals was transferred to plastic petri dishes (80 mm), pre-coated with 0.2% BSA, at  $10^7$  cells/ml in 3 ml complete medium at 37°C for 1 hour(27). Non-adherent cells were transferred to plates pre-coated with rabbit anti-mouse  
(kappa) (10  $\mu$ g/ml) and placed at 4°C for 1 hour. A  
second panning was performed on plates pre-coated with  
20 goat mouse Ig (Tago, Burlingame, CA). Recovery after this double panning procedure generally was 25 to 30% of the nucleated cells. In later experiments, T cells were enriched by binding to nylon wool by methods well known in the art. B cell contamination was examined by  
25 immunofluorescence using fluorescent goat anti-mouse Ig antibodies (Tago). The B cell fraction was usually 5 to 7%. The cell suspension enriched for T cells was placed in round bottomed wells (200  $\mu$ l/well,  $5 \times 10^5$  cells/ml). Twenty  $\mu$ l of stimulating agent (50  $\mu$ g/ml) was added to  
30 the wells. After 3 days of culture, 1.5  $\mu$ Ci of [methyl  $^3$ H]-thymidine was added to each well. After this 18 hour pulse, cells were harvested on glass fiber filters and incorporated radioactivity measured by scintillation counting. All measurements were performed in  
35 quadruplicate and the data are expressed as the mean cpm  $\pm$  SEM.

The results of the in vitro stimulation are shown in Table II. T cells from mice treated with DC-38C13 responded better to the idiotype protein than mice treated with idiotype KLH conjugates in CFA, or control mice.

PROLIFERATIVE RESPONSES IN LONG-TERM  
SURVIVING ANIMALS\*

10	[ <sup>3</sup> H] THYMIDINE INCORPORATION (cpm ± SEM	38C13 IgM	38C13- Sephadex	IL2	Unrelated IgM
15	DC-38C13 23,451 ± 1286 <sup>§</sup>	6,787 ± 376 <sup>§</sup>	10,071 ± 563 <sup>§</sup>	1,414 ± 160	
20	id. KLH 1,084 ± 127 in CFA	11,271 ± 898 <sup>§</sup>	2,166 ± 527 <sup>#</sup>	4,805 ± 363 <sup>"</sup>	
25	- <sup>†</sup> 1,453 ± 661	2,996 ± 243	850 ± 302	2,195 ± 395	

Table 2. \*Enriched splenic T cells were cultured in vitro during 3 days in the presence of 38C13 IgM. 38C13IgM coupled to Sephadex beads, IL2 containing medium or soluble unrelated IgM. Stimulation was measured by the degree of <sup>3</sup>H-thymidine incorporation during the last 18 hours of culture. <sup>†</sup>Normal C<sub>3</sub>H/He mice were used to purify unprimed T cells. <sup>§</sup>Significant at p<0.001 according to t-test with unrelated IgM. <sup>§</sup>Significant at p<0.001 according to t-test compared with unprimed T cells. <sup>#</sup>p>0.05 according to t-test compared with unprimed T cells. <sup>"</sup>Significant at p<0.002 according to t-test compared with unprimed T cells.

The T cell enriched spleen cells were also used in a conventional cell mediated cytotoxicity assay as follows:

45 Tumor cells were labelled with L-[4,5-<sup>3</sup>H] leucine (Amersham) and were placed in U-bottomed microwells at 1

X  $10^6$  target cells (100  $\mu$ l). Effector cells (enriched splenic T cells from surviving animals) were added at a ratio of 100/1, 50/1, 25/1, 6.25/1, 3/1, 1.5/1 in triplicate, the final volume being 200  $\mu$ l. The cells  
5 were sedimented by gentle centrifugation and the plates were incubated at 37°C in a moist atmosphere containing 5% CO<sub>2</sub> for 16 hours. Fifty microliters of supernatant was used to count the released radioactivity. Spontaneous and maximum release was determined by adding 100 $\mu$ l  
10 complete medium or 100  $\mu$ l 0.1% NP40 instead of T cells. Specific cytotoxicity was calculated according to the following formula:

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Cytotoxicity by immune lymphocytes was measured the day of T cell isolation and after a 3 day stimulation in vitro with 38C13-coupled sepharose beads. Unstimulated T cells were not able to lyse the 38C13 target cells. However, after stimulation in vitro, specific lysis of 38C13 tumor cells by DC-38C13 primed T cells was 21% versus 11% by 38C13-KLH stimulated cells (at an effector target ratio of 100/1 in a 16 hour incubation assay).

#### EXAMPLE V

##### Residual Tumor Cells

The presence of residual tumor cells in the spleen of long-term survivors was traced out by immunohistology and in vitro culture. Cryostat sections were fixed in acetone and stained with the monoclonal antibodies followed by streptavidin-horseradish peroxidase and diaminobenzidine tetrahydrochloride. Frozen sections of the spleen from long-term surviving animals were stained with biotinylated monoclonal anti-idiotypic antibodies. These antibodies had previously been tested on tumor



invaded spleen-sections where they stain both surface and cytoplasmic idiotypic IgM. No residual tumor cells could be detected in serial sections of the spleen of long-term survivors. B lymphocytes isolated from the spleen were  
5 cultured in vitro in enriched and conditioned medium. Although the 38C13 tumor cells used in this study are adapted to in vitro growth, none of the cultures showed outgrowth of tumor cells. Finally, B cells were transferred to irradiated syngeneic naive animals. No  
10 growth of tumor was observed during the 6 month observation time. These data indicate that no residual tumor cells remained in spleens of long term survivors.

## EXAMPLE VI

15 Alternative Preparation of Dendritic Cells

Spleens were perfused with a solution of 100 units/ml collagenase (CLS III, Worthington, Cat. Nr 4182, 1g/vial.) in 10 X Hanks Balanced Salt Solution (HBSS),  
20 (diluted from 10 X HBSS; Gibco Cat. Nr. 310-4065A/J.), (pH 7.2. adjusted with sterile GIBCO 7.5% bicarbonate). The cells were collected in a tube containing RPMI medium with 10% FCS. The remaining debris were incubated for 15 minutes in a solution of 400 units/ml collagenase, in the  
25 incubator. The solution was pipetted about 50 times to collect most of the cells. All the cells were pooled and pelleted.

Bovine Serum Albumin was prepared as follows:  
30 BOVUMINAR COHN FRACTION V powder (Armour Pharmaceutical Company, Kankakee, Illinois) was kept at 4°C in a dessicator. In a glass beaker (closed with aluminum foil), 65 ml distilled water; 186 ml PBS, 29 ml 1M NaOH and 107g BSA were combined and kept at 4°C for 24 hours  
35 without stirring. Density was checked in a refractometer and maintained between 1.3855-1.3865. If the solution was too dense, it was stirred very slowly and cold PBS

was added. It was filtered and kept at 4°C. The pellet was resuspended in a solution of cold BSA and centrifuged at 10,000 g; about 2 mls of serum-free medium was added on the top of the gradient. Low density cells were  
5 collected and represented about 10% of spleen cells.

The cells were cultured in 10% FCS-containing medium for 2 hours in 100 mm culture dishes. Non-adherent cells were washed out by vigorous pipetting (15 minutes per  
10 plate). Cells were again incubated for 1 hour in serum-free medium and non-adherent cells were removed by gentle pipetting. The remaining adherent cells were cultured overnight in 10% FCS-containing RPMI. The next  
15 day, the non-adherent cells were collected by gentle pipetting.  $2 \times 10^5$  to  $5 \times 10^5$  dendritic cells were obtained per spleen.

Although the invention has been described with reference to the presently-preferred embodiment, it  
20 should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

## WE CLAIM:

1. A method of inducing an effective immune response in a mammal to pathogenic lymphocytes expressing  
5 an idiotype protein on the cell membrane, comprising the steps of:
  - a) exposing dendritic cells in vitro to idiotype protein from said pathogenic lymphocyte to  
10 obtain idiotype pulsed dendritic cells; and
  - b) injecting said idiotype pulsed dendritic cells into said mammal.
- 15 2. The method of claim 1 wherein said pathogenic lymphocytes are tumor cells.
3. The method of claim 2 wherein said tumor cells are lymphoma cells.
- 20 4. The method of claim 2 wherein said lymphoma cells are B lymphoid cells.
5. The method of claim 1 wherein said idiotype protein is immunoglobulin.
- 25 6. The method of claim 1 wherein said idiotype protein is a fragment of an immunoglobulin bearing an idiotype.
7. The method of claim 1 wherein said mammal is a  
30 human.
8. Idiotype pulsed dendritic cells comprising dendritic cells which have been exposed in vitro to idiotype immunoglobulins.

1 / 2

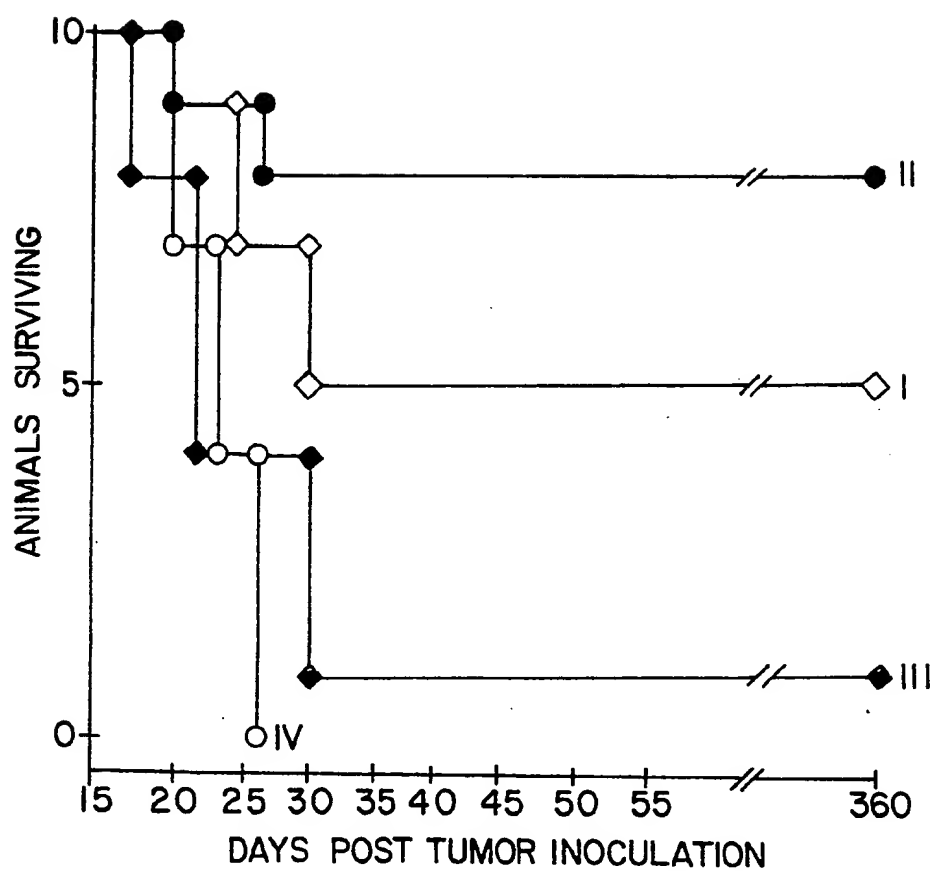


FIG. 1

SUBSTITUTE SHEET

2 / 2

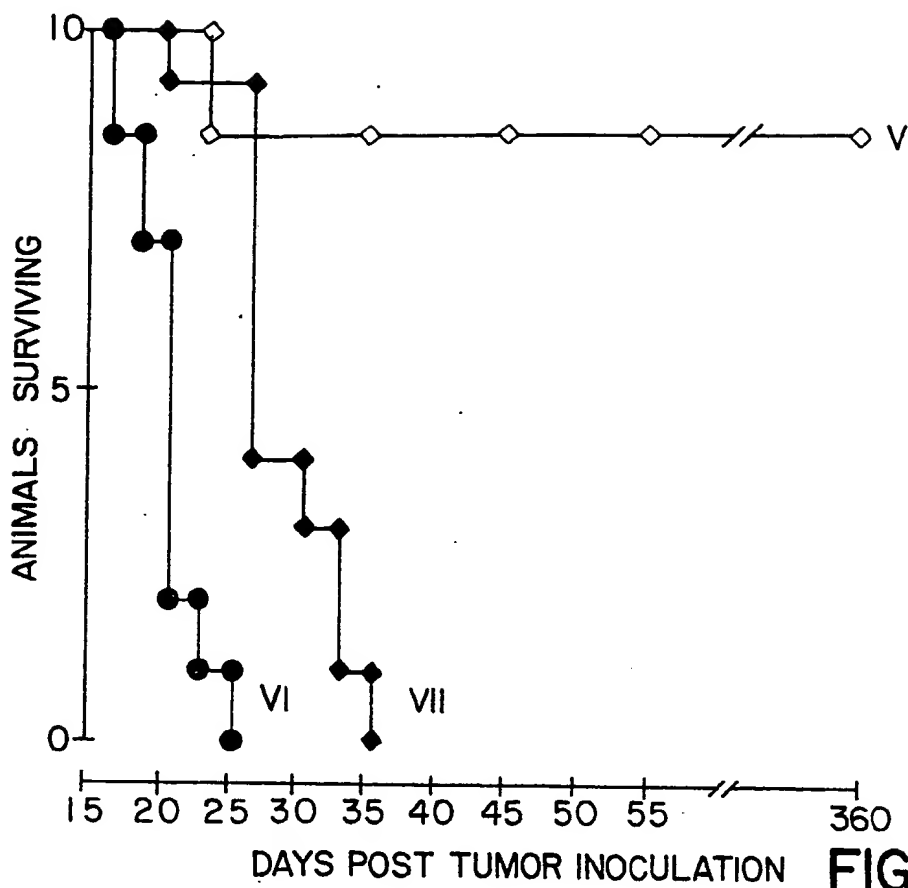


FIG. 2

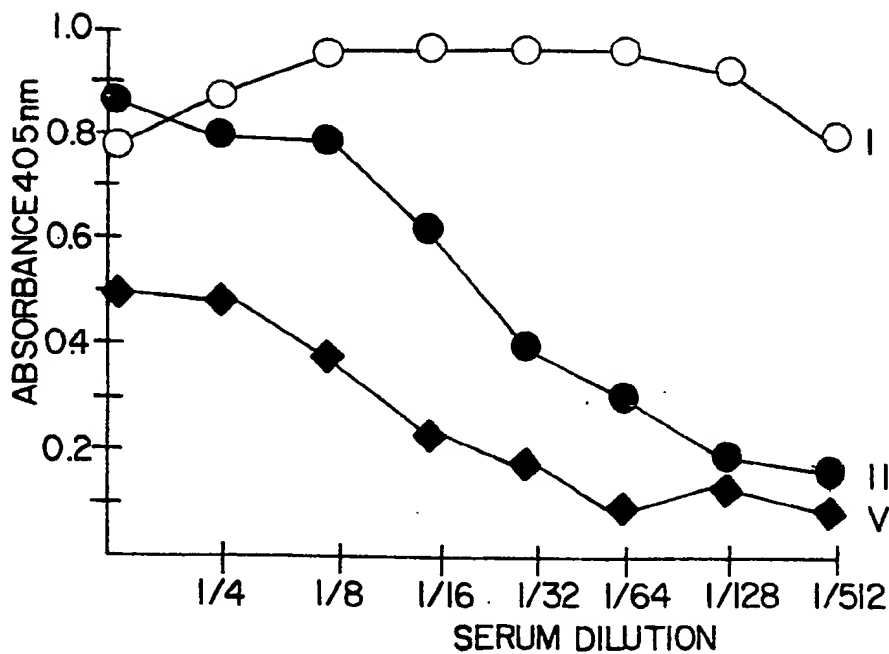
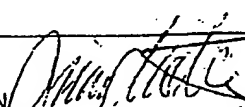


FIG. 3

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/01683

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>5</sup> : A 61 K 39/00, C 12 N 5/08		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>5</sup>	A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> *		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	Proc. Natl. Acad. Sci., volume 82, December 1985, (Washington, DC, US), M. Francotte et al.: "Enhancement of antibody response by mouse dendritic cells pulsed with tobacco mosaic virus or with rabbit anti-idiotypic antibodies raised against a private rabbit idio type", pages 8149-8152 see pages 8149-8152, in particular page 8151, left-hand column, line 43 - end cited in the application	8
A	The Journal of Immunology, volume 133, no. 1 July 1984, (Baltimore, US), K. Thielemans et al.: "Strategies for production of monoclonal anti-idiotypic antibodies against human B cell lymphomas", pages 495-501 see pages 495-501	8
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<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
20th June 1991	19. 08. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	miss T. MORTENSEN 	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 1-7, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv):

methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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